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Development and Characterization of a Clinically Compliant Xeno-Free Culture Medium in Good Manufacturing Practice for Human Multipotent Mesenchymal Stem Cells

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ABSTRACT

Human multipotent mesenchymal stem cell (MSC) therapies are currently being tested in clinical trials for Crohn's disease, multiple sclerosis, graft-versus-host disease, type 1 diabetes, bone fractures, cartilage damage, and cardiac diseases. Despite remarkable progress in clinical trials, most applications still use traditional culture media containing fetal bovine serum or serum-free media that contain serum albumin, insulin, and transferrin. The ill-defined and variable nature of traditional culture media remains a challenge and has created a need for better defined xeno-free culture media to meet the regulatory and long-term safety requirements for cell-based therapies. We developed and tested a serum-free and xeno-free culture medium (SFM-XF) using human bone marrow- and adipose-derived MSCs by investigating primary cell isolation, multiple passage expansion, mesoderm differentiation, cellular phenotype, and gene expression analysis, which are critical for complying with translation to cell therapy. Human MSCs expanded in SFM-XF showed continual propagation, with an expected phenotype and differentiation potential to adipogenic, chondrogenic, and osteogenic lineages similar to that of MSCs expanded in traditional serum-containing culture medium (SCM). To monitor global gene expression, the transcriptomes of bone marrow-derived MSCs expanded in SFM-XF and SCM were compared, revealing relatively similar expression profiles. In addition, the SFM-XF supported the isolation and propagation of human MSCs from primary human marrow aspirates, ensuring that these methods and reagents are compatible for translation to therapy. The SFM-XF culture system allows better expansion and multipotentiality of MSCs and serves as a preferred alternative to serum-containing media for the production of large scale, functionally competent MSCs for future clinical applications. STEM CELLS TRANSLATIONAL MEDICINE *2012;1:750–758*

INTRODUCTION

The use of mesenchymal stem cells (MSCs), also known as marrow stromal cells [1] and mesenchymal progenitor cells [2, 3], in the clinical arena to treat different diseases is advancing rapidly, as reflected by the increasing number of publications and clinical trials. Initial clinical trials with MSCs were conducted in patients with osteogenesis imperfecta [4], metachromatic leukodystrophy, and Hurler syndrome (mucopolysaccharidoses) [5]. Other diseases for which clinical trials using MSCs have been initiated are graft-versus-host disease, Crohn's disease, repair of skeletal tissue, amyotrophic lateral sclerosis, chronic spinal cord injury, nonhealing chronic wounds, vascular disease, coronary artery disease, and myocardial infarction. Currently, the largest clinical trials with MSC are in patients with cardiac disease [6, 7]. Most clinical trials using MSCs have derived these cells from bone marrow [8] with limited ex vivo expansion in serum-containing culture media (SCM). Despite using cells cultured in SCM, clinical trials of MSCs so far have shown no adverse reaction to allogeneic versus autologous MSC transplants, suggesting that culture-expanded MSCs did not have major histocompatibility complex (MHC) class II cell surface markers, but rather only MHC class I and no costimulator molecules [9, 10]. However, the risk of undefined components present in SCM or serum-free medium (SFM) formulated with ingredients of animal origin (xenogeneic), such as bovine serum albumin, porcine insulin, or bovine transferrin, pose a long-term safety risk. Hence, expanding and preparing MSCs under good manufacturing practice (GMP) conditions using xenogeneic (xeno)-free culture media, along with cell attachment solution, passaging method, and cryopreservation procedure, are critical.

Given the rapid advancement of the field, cells expanded in xeno-free media with defined components with specific quality characteristics that are safe for human clinical use and prepared in GMP conditions are expected to be used in future clinical studies regulated by the U.S. Food and Drug Administration (FDA). The FDA has issued guidance in the form of a Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) information for Human Somatic Cell Therapy Investigational New Drug Applications (IND) (April 2008) and in the 21 CFR Part 1270 and 21 CFR Part 1271 regulations. In continuation of the FDA guidelines and in an effort to meet the safety requirements, generation of clinical-grade cells is an important first step toward a wide range of possible future treatments. Toward this goal, we have developed a GMP-manufactured human MSC serum-free culture medium that could be used to generate large doses of cells required for autologous or allogeneic transplants [11]. Although the use of serum-free culture medium provides standardized and defined culture conditions compared with serum-containing medium, it still contains bovine serum albumin and other animal-derived components [12].The next logical step is the development of a better defined, completely xeno-free culture medium. In addition, this medium should enable the primary isolation and continual expansion of MSCs to allow the generation of clinical cell doses under a completely xeno-free workflow. This study reports the development of such a GMP-manufactured xeno-free culture medium with a proprietary mix of proteins, including human serum albumin, lipoproteins, recombinant human growth factors, and trace elements that are free of any nonhuman or animal origin components, and thus sets the stage for future MSC therapeutic applications.

Materials and Methods

Culture Expansion

Human bone marrow-derived MSCs (BMSCs) were cryopreserved at passage 4, thawed, and recovered in SCM consisting of low-glucose Dulbecco's modified Eagle's medium supplemented with 10% MSC-qualified fetal bovine serum (FBS), 2 mM L-glutamine, and 5 μg/ml gentamicin (unless specified, all reagents were from Life Technologies, Rockville, MD, http://www.lifetech.com). Upon reaching 70%–90% confluence, cultures were enzymatically treated with TrypLE Express, harvested, counted using a Countess automated cell counter, and seeded at 5×10^3 to 1×10^4 cells per cm^2 in SCM or StemPro MSC SFM XenoFree (serum-free and xeno-free culture medium [SFM-XF]) (catalog no. A10675-01; Life Technologies) supplemented with 2 mM L-glutamine and 5 μg/ml gentamicin. For long-term expansion (up to nine passages), cells were passaged every 4–6 days For SFM-XF cultures, vessels were precoated with a 1:100 cell attachment solution, CELLstart substrate in Dulbecco's phosphate-buffered saline (without calcium and magnesium), and the medium was replenished every other day. In addition, to demonstrate the potential of completely isolating and expanding BMSCs under xeno-free conditions, a limited number of single-donor isolation and expansion studies using fresh bone marrow aspirate were performed. To this end, BMSCs were isolated using SFM-XF supplemented with 2.5% pooled human AB serum, cryopreserved in the isolation medium supplemented with 10% dimethyl sulfoxide (DMSO), and then, upon thawing, continually propagated in SFM-XF (without the addition of human serum). CELLstart substrate was used for primary isolation and all subsequent passages.

Three lines of adipose-derived stem cells (ASCs), designated ASC12, ASC21, and ASC23, which have been characterized previously [13–16], were expanded for two passages in standard SCM composed of minimum essential medium-α supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. For multipassage expansion, cells were seeded at 2×10^3 cells per cm^2 in SCM or SFM-XF in conjunction with CELLstart substrate and grown for seven additional passages, for a total of 49 days in culture. The cells were harvested at weekly intervals using 0.125% trypsin and 0.01% EDTA and counted using a hemocytometer. The medium was changed between passages once for SCM and three times for SFM-XF.

Cell Proliferation Assay

To identify growth factors necessary to support cell proliferation in a serum- and xeno-free environment, cryopreserved passage 4 MSCs were thawed, expanded for a single passage in SCM, harvested using TrypLE, and seeded into 96-well black-walled tissue culture-treated plates (Corning Enterprises, Corning, NY, http://www.corning.com) at 1×10^4 cells per cm^2 in a growth factor-deficient SFM-XF supplemented with various combinations of recombinant human platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1). After 4 days, cell proliferation was measured using the CyQuant NF cell proliferation assay in a Safire^2 microplate reader (Tecan, Männedorf, Switzerland, http://www.tecan.com).

Karyotype Analysis

After serial expansion in SFM-XF, the BMSCs were seeded into T25 flasks, grown to mid-logarithmic stage, and shipped to Cell Line Genetics (Madison, WI, http://www.clgenetics.com) for karyotype analysis. Evaluations were performed to unveil any gross chromosomal abnormalities.

Immunophenotype Analysis

ASCs were enzymatically dissociated with Liberase (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com) and trypsin/EDTA, filtered through a cell strainer (70 μm; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com), rinsed, resuspended in wash buffer, and stained with prelabeled CD24, CD29, CD73, CD117, CD133, CD146, CD200, CD271, human leukocyte antigen HLA-DR (Abcam, Cambridge, MA, http://www.abcam.com), CD34, CD44, CD45, CD105, HLA-ABC (Dako, Glostrup, Denmark, http://www.dako.com), CD90, ABCG2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), and Stro-1 (Millipore, Billerica, MA, http://www.millipore.com) antibodies; for controls, matching isotype antibodies were used. Cells were then rinsed, treated with LIVE/DEAD Reduced Biohazard Viability/Cytotoxicity Kit #1, and fixed with 1% buffered formaldehyde. Cells were analyzed (1×10^4 events per run) with FACSCanto flow cytometer (BD Biosciences), and data were processed with the BD FACSDiva software (BD Biosciences). Gating based on bivariate forward versus side scatter plots was used to eliminate cellular debris, and the threshold for positive counts was determined as the

upper 5th percentile of intensity distribution in the control stains.

BMSCs were enzymatically dissociated with TrypLE, rinsed, and resuspended in wash buffer. The cells were formaldehyde fixed and washed before the permeabilization step with 0.1% Triton-X in phosphate-buffered saline. The cells were then incubated with the prelabeled antibodies CD14, CD19, CD34, CD45, CD73, CD105, and HLA-DR, as well as with CD90 (Biolegend, San Diego, CA, http://www.biolegend.com). Cells were washed and nuclear stained with 1 μM FxCycle Violet in wash buffer for determination of DNA content. The analysis was performed using an LSRII flow cytometer (BD Biosciences) under the control of the DIVA 6.1 software (BD Biosciences). Several sets of controls were used to ensure accurate results. They included single-color compensation to correct for spectral overlap between the selected fluorochromes, fluorescence-minus-one set up to define positive and negative populations, and the biological control. Information on culture expansion of human bone marrow-derived MSC and gated flow cytometry data for BMSCs can be accessed in supplemental online Figures 1 and 2. In addition, the sample set up, surface marker list, and fluorochromes can be reviewed in supplemental online Tables 1 and 2.

Gene Expression Analysis

RNA was isolated from frozen cell pellets using the RNeasy kit (Qiagen, Valencia, CA, http://www.qiagen.com) and further purified of contaminating genomic DNA using the DNA-free kit. The RNA was precipitated and quantified spectrophotometrically, and its purity was assessed by gel electrophoresis. Sample amplification and biotinylated cRNA was prepared and generated from 250 ng of total RNA using the TotalPrep RNA amplification kit (Illumina Inc., San Diego, CA, http://www.illumina.com) according to the manufacturer's protocol. Array hybridization to the Sentrix Chip Array (Human WG-6) (Illumina, San Diego, CA, http://www.illumina.com) and processing procedures were performed according to protocols provided by Illumina.

Differentiation

The BMSCs expanded in SCM or SFM-XF were seeded in StemPro Adipogenesis, Osteogenesis, and Chondrogenesis differentiation media, following the manufacturer's protocols, for 14–20 days. The progress toward specific lineages was assayed with 0.5% Oil Red O (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) staining for adipogenesis, by alkaline phosphatase (Leukocyte Alkaline Phosphatase Kit; Sigma-Aldrich) for osteogenesis, and by Alcian blue staining for chondrogenesis, as described previously [17]. The chondrogenic pellet was further analyzed for collagen II by immunohistochemical detection, using 3,3′-diaminobenzidine as a chromogen and Gill's hematoxylin as a counterstain [18].

Statistical Analysis

Data are presented as mean $\pm$ SEM. The differences between samples were assessed using analysis of variance followed by post hoc tests or a *t* test, and the statistical significance was assigned to $p < .05$. The procedures were carried out with the aid of SPSS 17 software package (SPSS, Chicago, IL, http://www.spss.com).

Results

Cell Expansion

The development of SFM-XF was based on earlier studies that revealed that a set of growth factors, including PDGF-BB, bFGF, and TGF-β1, had a promoting effect on human BMSC proliferation [12]. As shown in Figure 1A, any individual or double combination of growth factors provided enhanced expansion of human BMSCs compared with a growth factor-deficient serum-free medium; however, a combination of all three growth factors proved to be superior. To assess the effect of SFM-XF on morphology of cells, the BMSCs were propagated up to 45 days (passage 9 [P9]) and analyzed using phase contrast microscopy. When expanded in xeno-free culture conditions, the cells exhibited small, spindle-shaped morphology, whereas in a standard serum-containing environment, they had a flattened, fibroblast-like morphology (Fig. 1B). The same morphological pattern could be observed with ASCs that were isolated and propagated in xeno-free conditions for 35 days (P7) (data not shown). When the BMSCs were cryopreserved in SFM-XF with 10% DMSO, the cells exhibited good recovery, viability, and expansion (data not shown). It should be noted that the CELLstart substrate is a necessary requisite for long-term xeno-free cultures. In its absence, the cells will adhere poorly and fail to proliferate (data not shown).

The potential for SFM-XF to support the growth of MSCs was extensively evaluated using three ASC lines, ASC12, ASC21, and ASC23. Comparison of their average growth in SFM-XF with their growth in the serum-containing medium system revealed the superiority of SFM-XF. Not only did the cumulative cell number after 49 days (P7) in xeno-free culture surpass that in serum-containing medium by more than 30-fold, but a higher cell yield was already observed after 7 days (P1) and in each subsequent passage (Fig. 1C). Analysis of the cell doubling time demonstrated that the xeno-free system supported a significantly faster rate of growth until the fourth week. Following passage at day 20, the cells proliferated at a comparable rate in both conditions. We also observed that the xeno-free system had noticeable differential effect on growth rate of individual cell lines as compared with the serum-based system (Fig. 1D). Under standard growth conditions with SCM, cells accumulated at a linear rate for 49 days. Under xeno-free conditions, however, one of the cell lines (ASC12) grew substantially more slowly, especially toward the end of culture period, and the growth of another cell line (ASC21) noticeably slowed down during the last 2 weeks. Only ASC23 maintained a stable proliferation rate following the rapid expansion during the first 3 weeks. Analysis of the doubling times derived from individual cell lines confirmed the effect of xeno-free medium on the initial phase of accelerated growth and the selective slow-down in the later passages. In addition to ASCs, a limited number of BMSC cultures were isolated, established, and maintained under xeno-free conditions, and assayed for growth and differentiation during a long-term expansion. Results indicate that the xeno-free environment is capable of maintaining proliferation and differentiation of BMSCs comparable to that of ASCs (supplemental online Fig. 3).

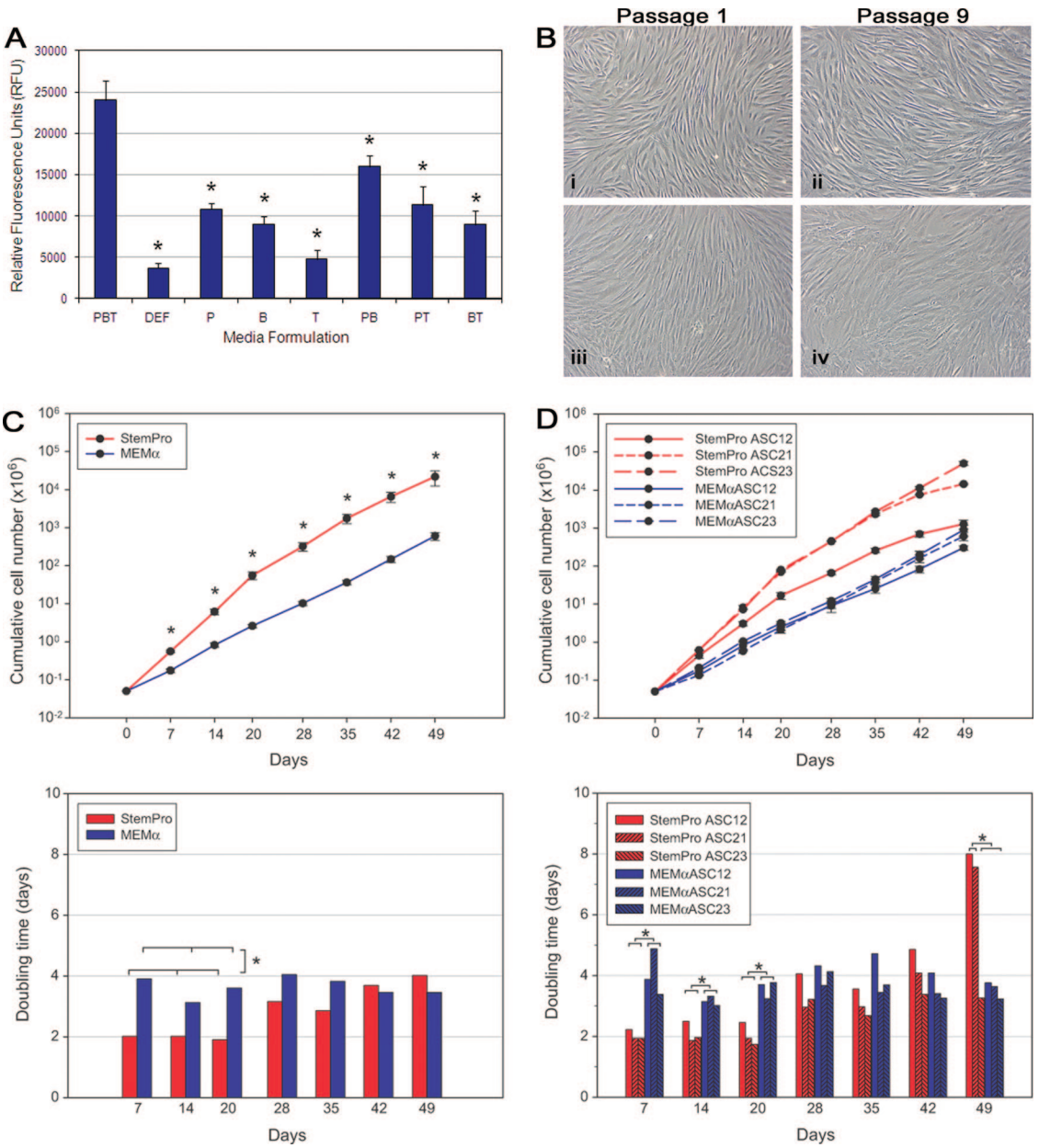

Figure 1. Expansion of ASCs and bone marrow-derived mesenchymal stem cells (BMSCs) in serum-free and xeno-free culture medium (SFM-XF) conditions. **(A):** Fluorescence-based (CyQuant NF Cell Proliferation Assay) quantification of human BMSC proliferation in deficient SFM-XF supplemented with various combinations of platelet-derived growth factor-BB, basic fibroblast growth factor, and transforming growth factor-β1. $*$, Statistically significant difference ($p < .01$; $n = 12$). **(B):** Morphology of BMSCs cultured under xeno-free conditions **(Bi, Bii)** and serum-containing conditions **(Biii, Biv)** at passages 1 **(Bi, Biii)** and 9 **(Bii, Biv)**. **(C):** The average proliferation characteristics from three unrelated ASC lines that were simultaneously grown in SFM-XF and standard serum-containing culture medium conditions. $*$, Statistically significant difference ($p < .05$; $n = 6$). **(D):** Individual proliferation characteristic of the three investigated ASC lines expanded in SFM-XF (StemPro ASC12, StemPro ASC21, and StemPro ACS23) and serum-containing conditions (MEMαASC12, MEMαASC21, and MEMαASC23). $*$, Statistically significant difference ($p < .05$; $n = 2$). Abbreviations: ASC, adipose-derived stem cell; B, basic fibroblast growth factor; BT, basic fibroblast growth factor plus transforming growth factor-β1; DEF, deficient serum-free and xeno-free culture medium; MEMα, minimum essential medium-α; P, platelet-derived growth factor-BB; PB, platelet-derived growth factor-BB plus basic fibroblast growth factor; PBT, platelet-derived growth factor-BB plus basic fibroblast growth factor plus transforming growth factor-β1; PT, platelet-derived growth factor-BB plus transforming growth factor-β1; RFU, relative fluorescence units; T, transforming growth factor-β1.

Genomic Stability and Transcriptomic Expression

Since novel medium technologies can influence the stability of chromosomes and introduce gross abnormalities over an extended passaging of stem cell lines [19], a karyotype analysis was performed on metaphasic BMSCs expanded for 25 (P5) and 45 days (P9) under xeno-free conditions. The analysis revealed no gross chromosomal aberrations, thus suggesting that the cells preserved their normal genotype (Fig. 2A; data shown for P9).

To evaluate a possible genotypic modulation under the influence of SFM-XF, transcriptional analysis was performed. The transcriptomic analysis was carried out on early (P3), intermediate (P6), and late (P9) passage BMSC cultures and

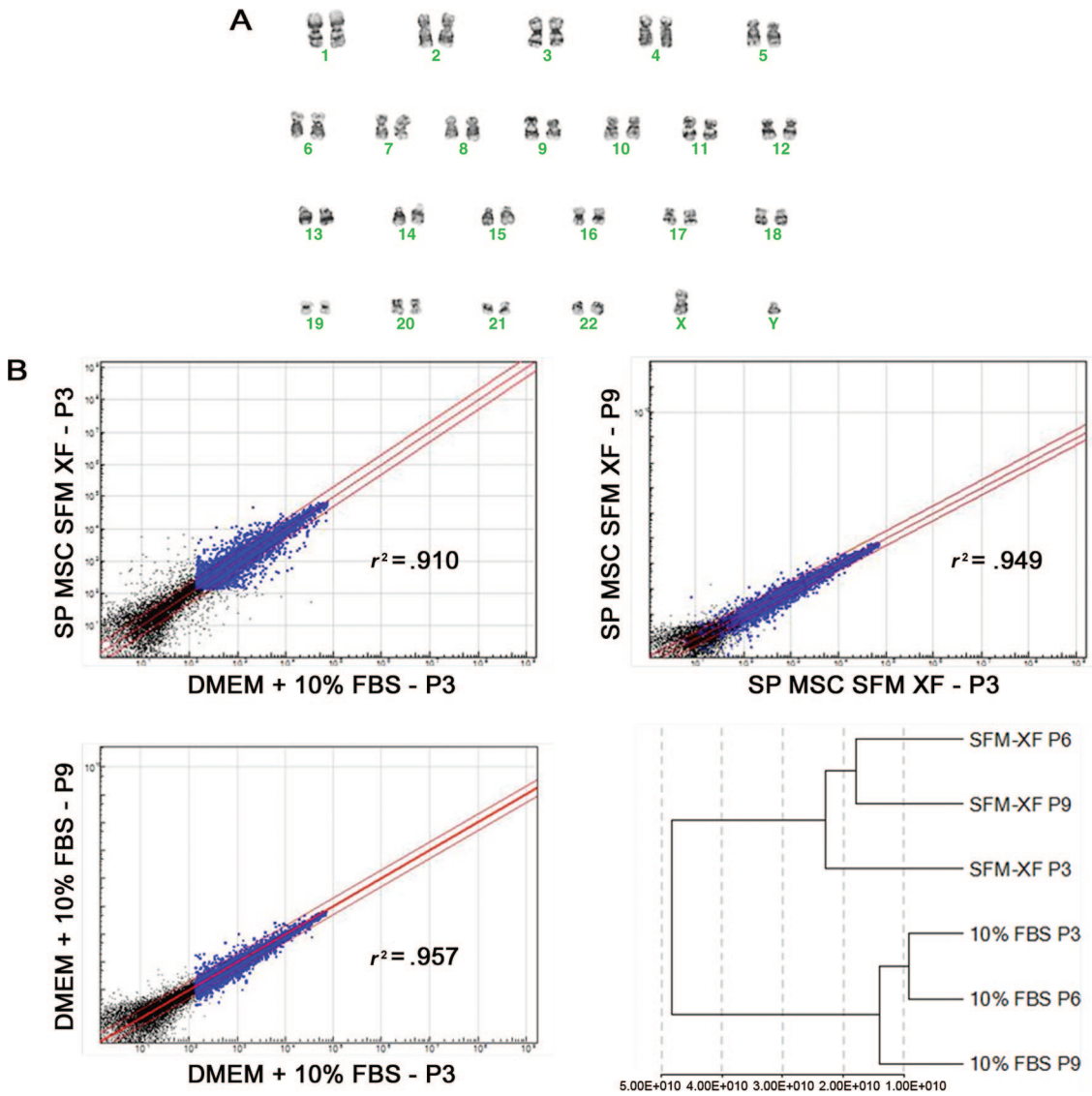

Figure 2. Karyotypic and transcriptomic analysis of bone marrow-derived mesenchymal stem cells (BMSCs) expanded in SFM-XF conditions. **(A):** Karyotypic characterization of BMSCs expanded in xeno-free conditions for 45 days (P9). **(B):** Pairwise comparisons of global gene expression patterns of BMSCs grown as xeno-free (StemPro MSC SFM-XF) and serum-containing (DMEM + 10% FBS) cultures for 15 and 45 days (P3 and P9) were determined using the HumanWG-6 v3.0 BeadChip Array. Evolutionary relationships based on global gene expression patterns of BMSCs grown in xeno-free and serum-containing conditions for 15, 30, and 45 days (P3, P6, and P9) were visualized by a phylogenetic dendrogram. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MSC, mesenchymal stem cell; P, passage; SFM XF, serum-free and xeno-free culture medium.

compared with results from cells grown in SCM. A global gene-expression analysis was carried out using the Illumina bead array containing 46,000 full-length and splice-variant transcripts from the Human RefSeq database. Differential gene expression detected above the 0.99 confidence level was considered for analysis. Interestingly, the pairwise scatter plot comparisons revealed that the media had a greater effect on the cells than did the duration of cultures (Fig. 2B). This is obvious from the comparison of early xeno-free and serum-containing medium systems, where the correlation parameter R^2 amounted to 0.91, whereas the R^2 values across the early and late passages were very similar for the xeno-free system and the serum-containing system, amounting to 0.949 and 0.957, respectively. Further analysis of genetic relatedness was done by constructing a neighbor-joining tree using maximum likelihood distances. The cladogram provided a clustering pattern that confirmed the initial conclusions based on pairwise comparison of transcriptional activation, and importantly revealed more advanced information (Fig. 2B). Grouping of samples on the basis of short Euclidean distances represents greater similarity than those far from each other. Again, the samples formed two distinct clades, according to medium systems, but surprisingly the standard serum-containing culture system appeared to maintain a homogeneous BMSC transcriptome with time.

Immunophenotyping

The immunophenotype profile was investigated in terms of similarity between the xeno-free and serum-based counterparts, as

Table 1. Adipose-derived stem cell flow cytometry

Marker	Passage 8 (% positive) SCM	Passage 8 (% positive) SFM-XF
CD24	0.9	3.2
CD29	75.4	89.4
CD34	2.4	1.6
CD44	98.5	98.7
CD45	1.4	1.4
CD73	66.8	66.1
CD90	90.3	92.6
CD105	97.9	87.9
CD117	1.0	1.4
CD133	1.4	1.8
CD146	2.1	1.9
CD200	2.3	1.8
CD271	1.7	1.5
ABCG2	1.4	1.9
HLA-ABC	94.1	95.1
HLA-DR	1.8	1.5
Stro-1	1.3	1.6

Abbreviations: HLA, human leukocyte antigen; SCM, serum-containing culture medium; SFM-XF, serum-free and xeno-free culture medium.

Table 2. Mesenchymal stem cell flow cytometry

Marker	Passage 5 (% positive)	Passage 9 (% positive)
SFM-XF		
$CD73^+/NEG^-$	99.3	99.9
$CD90^+/NEG^-$	96.4	100.0
$CD105^+/NEG^-$	96.5	99.9
$CD34^+$	0.1	0.6
SCM		
$CD73^+/NEG^-$	99.7	100.0
$CD90^+/NEG^-$	97.9	100.0
$CD105^+/NEG^-$	98.1	100.0
$CD34^+$	0.2	2.3

Abbreviations: NEG, mixed panel of negative biomarkers CD14, CD19, CD45, and HLA-DR; SCM, serum-containing culture medium; SFM-XF, serum-free and xeno-free culture medium.

well as temporal stability, using both ASCs and BMSCs. A comprehensive panel of 17 markers was used with ASCs (Table 1), and a panel of four markers that have previously been identified as essential to the MSC phenotype [20] was used with BMSCs (Table 2). Both types exhibited expression of cell surface markers in accordance with the data from previous studies based on serum-containing systems [21, 22]. The xeno-free environment did not seem to have any significant effect on the analyzed MSC epitopes with respect to serum-based condition or culture duration.

Maintenance of Functional Characteristics

To test the differentiation potential of human BMSCs expanded in SFM-XF, a classic mesoderm differentiation protocol was performed. As shown in Figure 3A, the cells expanded for nine passages retained the ability to undergo adipo-, chondro-, and osteogenic differentiation, as demonstrated by Oil Red O, Alcian blue, and alkaline phosphatase staining, respectively. Furthermore, a comparative analysis of the SCM and SFM-XF systems to support the chondrogenic specification of BMSCs from intermediate cultures was performed. The analysis of chondrogenic pellets for proteoglycan and collagen type II deposition demonstrated a greater extent of cartilage-specific extracellular matrix deposition with cultures in xeno-free medium (Fig. 3B) and demonstrated a full differentiation potential after 21 days (P6) (Fig. 3B). ASCs cultured in xeno-free medium also showed similar differentiation potential (data not shown).

Discussion

Several early-stage preclinical/clinical studies with human MSCs are under way despite considerable variability in cell sources, cell isolation methods, culture conditions for expansion, and lineage-specific differentiation. As the preclinical studies advance to the next stage, it is essential to develop regulatory friendly reagents that are xeno-free in nature to eliminate variations and make cell dosage vials similar to "pills" to generate transplant-ready cells. Given the rapid advances in the field, increased clinical use of MSCs is expected in the near future. However, in order to generate competent cells for therapy, cells need to be manufactured under GMP conditions, and cell culture scale-up steps would necessitate the use of a robust expansion medium that enables cell proliferation while retaining multipotency. Here, we describe the successful development of a novel xeno-free MSC culture expansion medium manufactured under GMP conditions to enable the production of clinical-grade MSCs. GMP manufactured medium removes uncertainties associated with batch-to-batch variations, is free of undefined animal components, and promotes the physiology and functionality of cells. The culture medium manufactured under GMP conditions meets preset specifications, and the production of medium follows validated standard operating procedures with traceability for questions of safety/performance evaluation.

As a first step in the process of development of a xeno-free culture medium, we developed an optimized serum-free medium, StemPro MSC SFM, that still contained some animal-derived ingredients [12]. Subsequently, we developed a complete xeno-free culture medium by using all USP-qualified ingredients of human or recombinant human origin for cell therapy. The growth factors PDGF-BB, bFGF, and TGF-β were a critical combination to achieve MSC proliferation and differentiation, whereas the absence of any one of the three growth factors impacted the performance of cells ability not only to expand but also to differentiate into osteo-, adipo-, and chondrogenic lineages. The combination of the three growth factors is critical [12], as is the optimization of other animal origin-free medium ingredients, such as human serum albumin, fatty acids, and trace minerals to ensure robust cell proliferation and lineage-specific differentiation to osteo-, chondro-, and adipocyte cell fates. Serum-free systems have been developed previously, but in many instances their performance was below that of serum-containing systems [23]. SFM-XF is a recently developed system, which involves not only medium but also a surface coating substrate (CELLstart) [12, 24]; it is the first such system that can compare truly favorably with the standard FBS-based products [25, 26]. If used in conjunction with an appropriate culture format such as microcarriers, it has a potential for large-scale expansion of cells, which is highly relevant for the prospective clinical applications [11].

A number of modifications involving mainly growth substrate and physical gaseous environments have been introduced in the standard protocols with the aim of further enhancing cell proliferation. The results obtained from systems using fibrin coating [27] or hypoxia [28, 29] indicate that there is room for modifications to suit a specific application. Yet from the vantage point of downstream clinical application, these approaches are burdened by potential hazards for cell therapy applications. The availability of a defined and xeno-free alternative would overcome such restrictions. Our current studies described here

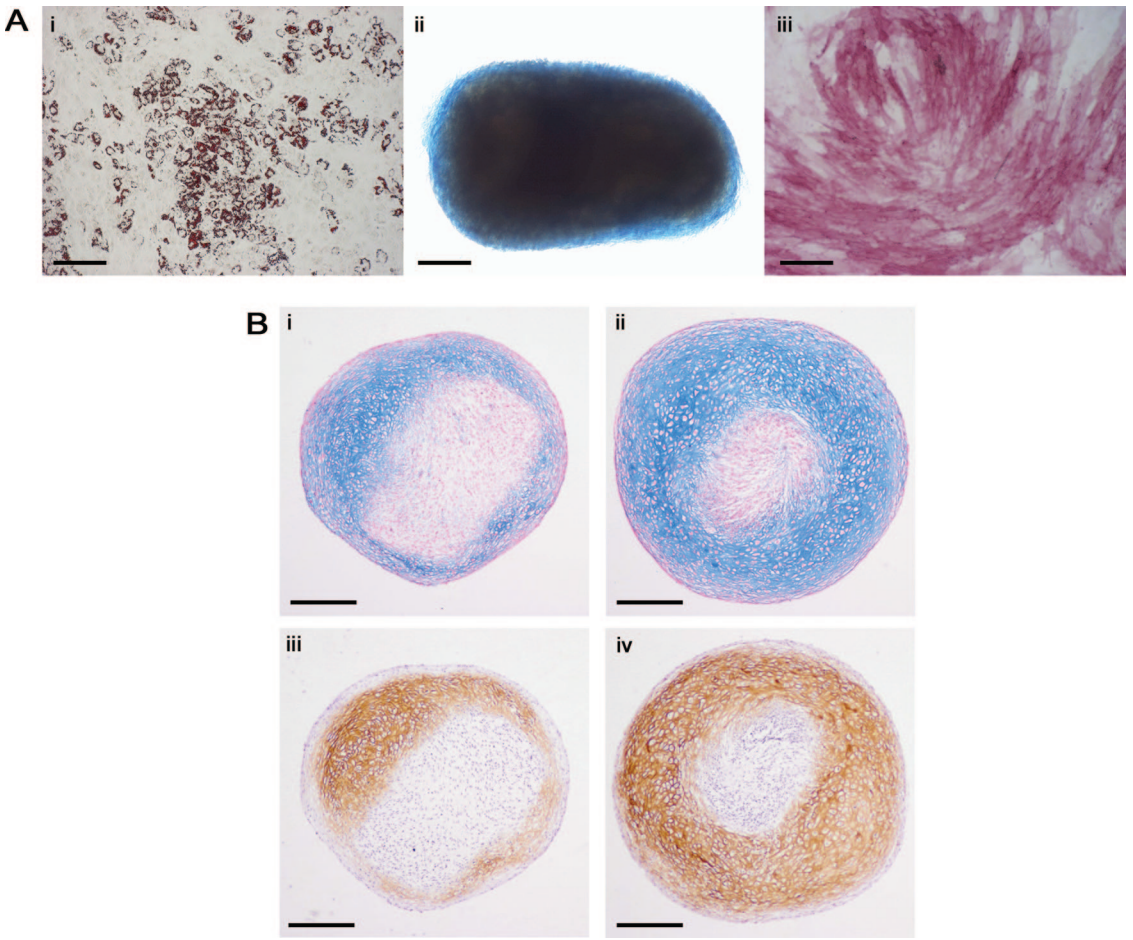

Figure 3. Differentiation potential and immunomodulatory properties of bone marrow-derived mesenchymal stem cells (BMSCs) expanded under serum-free and xeno-free culture medium conditions. **(A):** Adipo-, chondro-, and osteogenic differentiation revealed by Oil Red O **(Ai)**, Alcian blue **(Aii)**, and alkaline phosphatase **(Aiii)** staining, respectively, of BMSCs grown in xeno-free conditions for 40 days (P8). Scale bars = 100 μm. **(B):** Histochemical analysis using Alcian blue and nuclear fast red staining **(Bi, Bii)** and immunohistochemical detection of collagen type II using diaminobenzidine as chromogen and Gill's hematoxylin **(Biii, Biv)** as a counterstain of chondrogenic micromass cultures of BMSCs expanded in xeno-free conditions (P6) **(Bii, Biv)** and serum-containing conditions (P3) **(Bi, Biii)**. Representative micrographs from two independent experiments based on two different donors are shown. Scale bars = 100 μm.

confirm that the xeno-free formulation StemPro MSC SFM XenoFree, when used together with the defined substrate CELLstart, provides a better, preferred culture system option to enable a path for human mesenchymal stromal cells for cell therapy and clinical trials.

MSCs have a broad range of specific properties that are important for their clinical utility in cell-based therapies, regeneration, restoration of physiological environment, cell replacement, and tissue engineering [30]. They include differentiation potential, in vitro/vivo secretion of paracrine factors, revascularization ability, immunomodulatory property, and immunosuppression property. It has been suggested that some aspects of the phenotype may change during in vitro expansion [31–33]. Thus, it is important that new culture paradigms are designed to affect properties critical for a therapeutic effect.

In addition, MSCs exhibit immunomodulatory effects on various immune cells, such as T and B cells, dendritic cells, and natural killer cells, both in vitro and in vivo [34]. The immunosuppressive effect of MSC culture expanded in xeno-free culture media versus serum-containing media on T cells demonstrated a clear immunosuppressive functionality of the cells (data not shown), indicating the suitability of xeno-free medium for preparing such cellular populations for MSC-mediated cell therapy interventions.

Interestingly, the comprehensive gene expression study carried out with the cells cultured in xeno-free versus non-xeno-free culture conditions in this study is consistent with an earlier study that compared transcriptome profiles between cells cultured in traditional FBS-containing media and StemPro MSC SFM [12]. The global gene expression data generated with multipassage cells show two main clusters, cells cultured in FBS-containing media and cells cultured in SFM-XF. Despite the distinct clustering, pairwise analysis of cells in FBS versus cells in SFM-XF shows close correlation, with high R values. Importantly, pairwise comparison of cells at early passage versus late passage under both medium conditions demonstrates fairly high correlation, with values comparable between the two medium systems. The small subset of genes that do change with passage could be different for the two medium systems, but taken together with other data presented, it does not seem to have an impact on either proliferation or differentiation of MSCs. Based on these results, it can

be concluded that there is no significant drift in global gene expression with extended passage and like traditional medium systems; SFM-XF can maintain proliferation ability of MSC.

Conclusion

In summary, we have systematically developed a xeno-free culture medium using a set of experiments for human mesenchymal stromal cells isolated from bone marrow and adipose tissue. When used together with CELLstart xeno-free substrate, this culture system may enable the creation of MSC banks in a regulatory-friendly condition suitable for cell therapy. Maintaining MSC phenotype/functional properties is critical for clinical utility, and it is thus important that new culture systems be designed to retain the properties critical for a therapeutic effect. We were able to demonstrate from this study that the major functionality of the tested cells is well maintained in this xeno-free culture system. Furthermore, it is possible to extend the use of xeno-free culture media for large-scale cultures on microcarriers that can meet the increasing need for transplant-ready cells (in this case MSCs) in the regenerative medicine field.

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Author Contributions

L.G.C.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, provision of study material or patients, manuscript writing; S.Y.: conception and design, financial support, collection and/or assembly of data; V.Z.: conception and design, financial support, data analysis and interpretation, provision of study material or patients, manuscript writing; Z.Y.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, provision of study material or patients; U.L. and J.B.: conception and design, collection and/or assembly of data, data analysis and interpretation; S.E.B.: conception and design, financial support, administrative support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing; M.C.V.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

L.G.C., U.L., J.B., S.E.B., and M.C.V. contributed to this work while employed at Life Technologies, a publicly traded global company. U.L., J.B., S.E.B., and M.C.V. hold stock in Life Technologies.

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